TABLE 3. ^{125}I incorporation into iodothyronine fraction in thyroid

1st generation			2nd generation			3rd generation		
C	Mn	Mn/C	C	Mn	Mn/C	C	Mn	Mn/C
%	%		%	%		%	%	
Female								
8.92 (11.56)	11.05 (10.73)	1.24 (0.93)	6.18 (10.60)	7.05 (10.10)	1.14 (0.95)	5.85 (6.09)	7.09 (7.68)	1.21 (1.26)
Male								
5.18 (5.88)	3.82 (4.95)	0.74 (0.84)	9.08 (11.50)	6.12 (9.57)	0.67 (0.83)	7.45 (9.87)	3.42 (5.32)	0.46 (0.54)

C: Control, Mn: Mn treated.
The values represent the percentages of ^{125}I activity in T_3+T_4 fractions to total radioactivity on chromatograms. Values were obtained by using developing solvent system of *n*-butanol:ethanol:2N ammonium hydroxide=5:1:2 (v/v) and the values in parentheses were obtained by using a system of *n*-butanol : acetic acid : H_2O=4:1:2 (v/v).

some dwarfs showed ataxial motion, the brains of dwarfs were subjected to histological examination. Although the photographs are not shown, both control and dwarf neonates displayed regular lamination of nerve cells in the motor area of the cerebral cortex, and the dwarfs exhibited nerve cells which were stained lighter than the control. There was no critical difference in lamination of nerve cells of cerebellar cortex between the two groups. In the dwarfs, however, many swollen and lightly stained Purkinje cells in the cerebellar cortex were seen as well as an increased number of nerve fibers in the cerebellar medulla, there was no difference in stainability of the Nissl bodies of the nuclei cerebri in normal and dwarf mice.

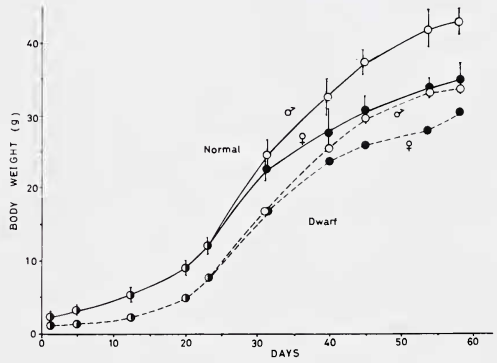

FIG. 1. Growth curve of normal and dwarf neonates. The normal group consisted of 13 male and 9 female mice from 2 litters and for the dwarfs, one male and one female. The vertical bar represents the standard deviation.

FIG. 2. A photograph of normal and dwarf neonates. The photograph shows a dwarf (upper) and a normal (lower) neonate on day 9 after birth.

DISCUSSION

Although symptoms caused by manganese deficiency [7] and neurological dysfunctions by excess manganese [8] have been well documented, knowledge of neonatal endocrinology related to excess manganese ingestion is scant. Previously, we stated that even with sufficient iodide supply, excess manganese can be goitrogenic in the female mouse thyroid, but not in the male mouse thyroid [5]. The present study provided additional confirming evidence to support the observation mentioned above. Moreover, goiters were always produced in three generations by continuous administration of manganese. However, the size of goiters did not increase from one generation to the next as shown in Table 1. Initially, we assumed that the condition of goiter would become more severe with each succeeding generation if genetic factors affected goiter formation by manganese. Judging from the result in Table 1, there was no such hereditary influence in the manganese effect.

Histological observations revealed that the goiter caused by manganese consisted of flattened epithelial cells with ample colloid in the lumen. This feature of colloid filled goiter suggests that the thyroid must be in a hypofunctional state, perhaps due to the second phase of Marine Cycle [9]. Therefore, it is likely that the use of colloid by epithelial cells was blocked by manganese in female mice. This speculation is supported by the fact that radioiodide activity in the intrathyroidal T_3+T_4 fraction was slightly but steadily higher in female mice with manganese treatment than in control mice (Table 3). This result suggests that in females, the *de novo* synthesized hormones remain in the lumen, and in males, the hormones are promptly released from the lumen.

From the present study, it is not known whether TSH levels were altered by manganese. We attempted to use a rat-TSH antibody to measure mouse TSH level by radioimmunoassay, but the rat-TSH antibody did not react with the mouse TSH. Buthieau and Autissier reported that the serum TSH level was reduced in manganese injected rats [4]. If one could extend this evidence to mice, excess manganese may depress the thyroid and the pituitary functions. Na^+, K^+-ATPase, which suplies energy to epithelia for colloid endocytosis, was not significantly inhibited by manganese *in vitro* (authors, unpublished). This evidence was compatible with the fact that the prolonged oral administration of manganese only slightly affected the ratio of ^{125}I activity in tissue to that in serum [5]. Therefore, this feature was entirely different from the acute parenteral treatment [3]. At present, the cause of the sex difference in the manganese effect on colloid accumulation is not clear, but in castrated male mice, goiter was induced by manganese, suggesting levels of male and female hormones with manganese may somehow bring about the formation of goiter [5].

Serum T_4 levels in non-pregnant females, dams and the plasma of neonates were not significantly affected by manganese administration. Perhaps the manganese effect was mild so that regulatory processes to maintain homeostasis of the hormone level could operate properly in all ages of animals examined. Since the presence of manganese transferrin has been demonstrated in sera from several species [10–12], a similar carrier protein may exist in the mouse to reduce the toxic effects of an overload of manganese.

In the third generation of manganese treatment, dwarfs were born, but there is no information suggesting that this was specifically due to manganese treatment. When the manganese content in milk of the treated dams was measured, it was not significantly different from the control milk (data not shown). The course of dwarfism may occur during the period of pregnancy. Dwarfs could grow, but showed ataxial motion. We do not know whether the ataxia in dwarfs mimics Parkinsonism in humans caused by manganese intoxication [8]. When organ distribution of manganese was examined, the level of manganese content in the entire brain was not high compared with other organs [5, 16]. However, high amounts of endogenous manganese was found in the hypothalamus region of rats [13], indicating that manganese could be localized in narrow regions to cause aberrations in the nervous system.

Donaldson *et al.* [14] have shown that manganese can exist *in vivo* as either an oxidant or an antioxidant, depending on its valency state; Mn^{2+} may reduce norepinephrine levels in brain regions

whereas Mn^{3+} enhances lipid peroxidation and formation of free radicals. Our recent study on partition of Mn^{2+} and total manganese in rat organs showed that only a very small fraction of the total manganese remained in the form of Mn^{2+} in most organs after manganese chloride administration [16]. These data suggest that transvalency of manganese by biological systems is very active. Histological study of the brain region of dwarfs exposed to manganese in early developmental stages showed no drastic alterations. It is likely that the effect of excess manganese on the perinatal nerve systems may not be drastic but mild and latent in nature.

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Neuroendocrine Regulation of the Development of Seasonal Morphs in the Asian Comma Butterfly, *Polygonia c-aureum* L.: Is the Factor Producing Summer Morphs (SMPH) Identical to the Small Prothoracicotropic Hormone (4K-PTTH)?

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ABSTRACT—In the butterfly, *Polygonia c-aureum* L., a physiological mechanism underlying the photoperiodic control of seasonal-morph development involves a summer-morph-producing factor (SMPH). Amounts of SMPH present in the brain-extracts of *Polygonia* pupae as well as in those of the silkmoth, *Bombyx mori*, were evaluated by *Polygonia* pupal assay for SMPH.

The factors responsible for SMPH-activity in the *Polygonia* and *Bombyx* brain-extracts were heat-stable (95°C for 5 min), but they were thought to be peptide hormones since the brain-extracts became SMPH-inactive by hydrolyzing with trypsin (24 hr at 25°C). According to the Sephadex G–50 gel-filtration pattern of SMPH-activity, the ultimate molecular weight of SMPH was though to be 4500, which is almost the same size as the small prothoracicotropic hormone purified from the silkmoth, *B. mori* (4K-PTTH or bombixin: M. W. 4400). This finding is also supported by evidence in our present study that 2 (out of 4) Sephadex G–50 fractions judged as being SMPH-active showed 4K-PTTH-activity by *Papilio* pupal assay. However, the factor showing SMPH-activity was not thought to be identical with the one showing 4K-PTTH-activity in *P. c-aureum* since they were separated by reversed-phase HPLC.

INTRODUCTION

The Asian comma butterfly, *Polygonia c-aureum* L., exhibits seasonal dimorphism, i.e., summer and autumn morphs. Development of the seasonal morphs is governed by photoperiod and temperature exposure during the larval stages [1, 2]. A physiological system underlying the photoperiodic control of seasonal-morph development involves a neuroendocrine system of brain-corpus cardiacum-corpus allatum, which secretes a summer-morph producing factor (SMPH) in the early pupal stage [3, 4]. The factor showing SMPH-activity is present in the brains of *P. c-aureum* as well as in the brains of several other lepidopteran insects, *Bombyx mori*, *Papilio xuthus* and *Lycaena phlaeas daimio* [5]. The SMPHs could be extracted and precipitated in almost the same manner as has been demonstrated in the small and big prothoracicotropic hormones (4K- and 22K-PTTHs) of *B. mori* [5]; the PTTHs were extracted with 2% NaCl, but not with acetone and 80% ethanol, and were precipitated by ammonium sulfate at 80% saturation [6].

The present study was designed to determine whether the factors showing SMPH-activity are identical to the 4K- and/or 22K-PTTHs in lepidopteran insects, *P. c-aureum* and *B. mori*.

MATERIALS AND METHODS

Animals Larvae of *P. c-aureum* and *P. xuthus* were held in either transparent plastic containers of $\phi 9 \times 5\ cm^3$ or $19 \times 13 \times 5\ cm^3$ and exposed to short-day (SD) conditions alternating periods of 8-hr of light and 16-hr of dark. The

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rearing containers were placed in a cabinet at constant temperature (20°C or 25°C) and were illuminated by two 20 W white fluorescent tubes which were controlled by a 24-hr time-switch. During the light period, light intensity was provided at about 500 lux.

Larvae of *Polygonia* and *Papilio* were fed on leaves of *Humulus japonicus* and *Fagara ailanthoides*, whereas those of the silkmoth, *B. mori*, were fed on leaves of *Morus tiliaefolia.* Fresh leaves were provided daily.

Under the short-day condition, *Polygonia* larvae and pupae all developed into butterflies of autumn morphs, whereas those of *Papilio* entered diapause in the pupal stage.

Extraction of SMPH Brains were obtained from 0-day-old *Polygonia* pupae (4 to 12 hr after larval-pupal ecdysis) and the silkmoth, *B. mori*, by dissection in saline (0.9% NaCl) and stored at −85°C. Four hundred brains were grouped, homogenized with a Teflon homogenizer in ice-cold acetone (a total volume of ca. 4 ml), washed 3 times in 80% ethanol (a total volume of ca. 0.8 ml) and extracted 3 times with 2% NaCl (a total volume of ca. 0.6 ml) at 0°C. At each step, insoluble materials were removed by centrifugation at 12,000×*g* for 30 min. The brain-extract of 2% NaCl was heated in a boiling-water bath for 5 min, rapidly cooled and centrifuged at 12,000×*g* for 30 min. The supernatant was added to solid ammonium sulfate up to 80% saturation to precipitate the factor. Then the precipitate was dissolved in 0.1 M ammonium acetate (pH 7.02) and stored at −85°C. The solution was used as crude SMPH.

Trypsin-hydrolysis The crude SMPH was derived from 800 brains of 0-day-old SD-pupae, dissolved in 0.2 M ammonium acetate containing 0.01 M calcium chloride (700 μg protein/230 μl) and incubated with trypsin (7 μg/230 μl) for 20.5 hr at 25°C. Twenty-four hours later, the incubation mixture was heated in a boiling water bath (95°C) for 3 min, rapidly cooled and centrifuged again at 12,000×*g*. The supernatant was bioassayed for SMPH in addition to a control sample heated before incubation.

Sephadex G–50 gel-filtration The crude SMPH from 800 brains of *Polygonia* pupae or from 250 brains of the silk moth, *B. mori*, was applied on to a column (10.2×912 mm) of Sephadex G–50 (Pharmacia, superfine) and eluted with 0.1 M ammonium acetate (7.3 ml/hr·cm). Sample fractions consisting of 100 drops each (3.9 ml) were collected for about 15 hr, lyophilized and stored at −85°C.

Reversed-phase HPLC Sephadex G–50 *Polygonia* fractions showing SMPH-activity and a highly purified 4K-PTTH of *B. mori* (bombixin) [7] — a gift from Professor H. Ishizaki of Nagoya University — were dissolved in 100 μl of 0.2 M ammonium acetate, subjected to reversed-phase HPLC (Shimadzu LC–3A, Tokyo) on a column of Hi-Pore RP–308 (Toyo Soda, Tokyo). Linear gradient elution with 10–40% acetonitril in 0.1 M ammonium acetate was performed over 65 or 90 min. Sample fractions through reversed-phase HPLC were collected at 4- to 6-min intervals, lyophilized and stored at −85°C.

Bioassay of SMPH Five microliters of samples with/without brain-extracts or the highly purified 4K-PTTH of *B. mori* were injected into the abdomens of 0-day-old female *Polygonia* pupae (4 to 12 hr after larval-pupal ecdysis) at different doses (1- to 100-brain equivalents). The recipient pupae were allowed to develop at 25°C.

On the day of emergence, butterflies derived from the treated pupae were classified into one of the grades 0–4. An average grade score (AGS) for summer morphs, on which the SMPH-activity of the sample was evaluated, was obtained from the response of 6–20 insects. The classification was based on a gradient used to judge the color of the ventral side of the wings [4].

Bioassay of 4K-PTTH Five microliters of samples with/without brain-extract (1- to 100-brain equivalents) were injected into the abdomens of diapausing *Papilio* pupae (15 days after larval-pupal ecdysis at 25°C). The recipient pupae were placed at 25°C and observed to see whether or not they showed any sign of adult-development (eye-pigmentation). When the pupae exhibited this sign

of adult-development within 15 days after treatment, the test-sample was judged as showing 4K-PTTH-activity.

RESULTS

Fractional precipitation of SMPH-activity with ammonium sulfate

An extract from 200 brains of *Polygonia* SD-pupae (0-day-old) was made with 2% NaCl and fractionated by adding solid ammonium sulfate up to 0.8 saturation. Each precipitate was dissolved in distilled water and bioassayed using female *Polygonia* SD-pupae (0-day-old).

Figure 1 shows that an approximately half of the SMPH-activity present in the 2%-NaCl extracts was precipitated by ammonium sulfate between 0.5–0.65 saturation. SMPH-activity was mostly precipitated by ammonium sulfate at 0.8 saturation as has been demonstrated in the case of 4K- and 22K-PTTHs of *B. mori* [6, 7].

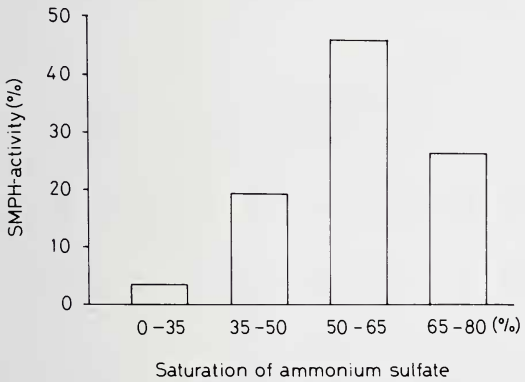

FIG. 1. Fractional precipitations of SMPH-activity with ammonium sulfate. Histograms show the percentages of SMPH-activity recovered from the precipitates of fractional precipitations.

Effects of heating and trypsin-hydrolysis on the SMPH-activity

To determine whether or not the factor responsible for SMPH-activity in *Polygonia* brain-extracts is heat stable and also is resistant to trypsin hydrolysis, a 2%-NaCl extract was prepared from 800 brains of *Polygonia* pupae. The extract was divided into 4 parts, heated in a boiling-water bath (95°C) for different time-periods (0, 1, 3 or 5 min), rapidly cooled and centrifuged. The supernatants were bioassayed for SMPH. In addition, an extract was made from 800 brains of *Polygonia* pupae in the same manner as above, hydrolyzed with trypsin for 24 hr at 25°C and bioassayed for SMPH along with a sample incubated without trypsin.

A high recovery (86%) of SMPH-activity was obtained from the heat-treated crude SMPH preparation (Table 1). However, no activity was detected in the trypsin-hydrolyzed extracts by *Polygonia* pupal assay for SMPH (Table 1).

The results indicated that the factor responsible for SMPH-activity of the *Polygonia* brain-extracts was stable upon heating but appeared to be a peptide hormone since it was hydrolyzed by trypsin.

Gel-filtration of the crude SMPH preparations through Sephadex G–50.

Crude SMPH was provided from either 800 brains of *Polygonia* SD-pupae or from 250 brains of the silkmoth, *B. mori*, lyophilized, dissolved in 400 μl (350 μl in *B. mori*) of 0.2 M ammonium acetate, applied to a column of Sephadex G–50 (Pharmacia, superfine), and eluted with the same solution (7.3 ml/hr·cm).

SMPH-activity was detected from 5 of 13 gel-filtrated fractions (tubes no. 11–15), in which *Polygonia* and *Bombyx* materials of M.W. 3500–5500 were thought to be collected (Fig. 2). Recoveries of the SMPH-activity by the gel-filtration through Sephadex G–50 reached 40–50% and 56–62% in the *Polygonia* and *Bombyx* crude SMPH preparations, respectively. Furthermore, two Sephadex G–50 fractions of the *Bombyx* crude SMPH (tubes no. 12 and 13) which showed higher SMPH-activity than the others were judged as being 4K-PTTH-active by *Papilio* pupal assay (Fig. 2).

The results indicated that the molecular sizes of the *Polygonia* and *Bombyx* SMPHs are about 4500 (3500–5500), which are almost the same size as the 4K-PTTH of *B. mori* (M.W. 4400) but are far smaller than the size of the 22K-PTTH (M.W. 22,000). However, the SMPHs could not be